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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 20 April 2004 with an application for Letters Patent number 532382 made by BLIS TECHNOLOGIES LIMITED.

Dated 5 August 2004.

PRIORITY DOCUMENT
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NEW ZEALAND
PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

TREATMENT OF MALODOUR

We, **BLIS TECHNOLOGIES LIMITED**, a New Zealand company of Level 10, Otago House, 481 Moray Place, Dunedin, New Zealand, do hereby declare this invention to be described in the following statement:

TREATMENT OF MALODOUR

FIELD OF THE INVENTION

This invention relates to methods of treating halitosis, and to the use of *Streptococcus salivarius* strains and compositions containing same in the prevention or treatment of halitosis.

BACKGROUND

Halitosis or bad breath is a common complaint characterised by the production of volatile sulfur compounds. The production of such compounds is generally associated with oral bacteria, particularly certain anaerobic species. These bacteria generally inhabit oral surfaces, and particularly periodontal pockets and the dorsa of the tongue surface.

The primary source of volatile sulphur compounds (VSC's) from the subgingival microflora is from microorganisms that can be both commensal and pathogenic. Previous culture-based studies have indicated that *Porphyromonas gingivalis, Prevotella intermedia* (both black pigmented species), *Fusobacterium nucleatum, Micromonas micros* (formerly, *Peptostreptococcus*), *Bacteroides* species, *Campylobacter rectus, Eikenella corrodens, Desulfovibrio* species, *Treponema denticola, and Eubacterium* species amongst others are responsible for the production of VSC's that contribute to halitosis (as summarized by Loesche WJ, Kazor C. Periodontol 2000. 2002;28:256-79. and Khaira N, Palmer RM, Wilson RF, Scott DA, Wade WG. Oral Dis. 2000 Nov;6 (6):371-5.). However, recent non-culture based studies have shown that there are certain species associated with subjects that are either healthy or afflicted with halitosis. *Atopobium pavulum, Eubacterium sulci, Fusobacterium periodonticum, Dialister*, a phylotype of streptococci, a phylotype of the uncultivated phylum TM7, and *Solobacterium moorei* appeared to be present in subjects with halitosis. By contrast, *Streptococcus salivarius, Rothia mucilaginosa (Stomatococcus mucilaginosus)*, and an uncharacterized *Eubacterium* (strain FTB41) were commonly detected only amongst healthy individuals (Kazor, C.E. et al., J. Clin Microbiol, Feb 2003, pp 558-563).

Over the years various methods have been developed and tried with varying success, to prevent or at least alleviate the problem of halitosis. Current treatments focus on anti bacterial regimes to reduce numbers of oral bacteria, or agents to mask or neutralise the

offensive odour. Oral rinses with chlorine dioxide have been shown to have some effect in the control of halitosis, but the levels of chlorine dioxide are in excess of accepted levels in drinking water, and are not recommended for ingestion. Current methods require complex physical, chemical or expensive regimes to be carried out and are typically only of short term effect, as the malodour-causing oral bacteria recover to former levels after treatment is stopped.

What is sought to treat halitosis is the replacement of the disease-causing organisms, with a non-virulent commensal microorganism. To serve as an effector strain in replacement therapy, the microorganism must be able to compete successfully with the pathogenic microorganism either via competitive action (eg for attachment sites), and/or antibiotic action, or inhibition by other metabolism-associated by-products.

In WO 01/27143 *S. salivarius* strains are identified which have utility in the treatment of dental caries caused at least in part by *S. sobrinus*. No activity was recorded against any anaerobic microorganisms. Moreover, the treatment of halitosis is nowhere contemplated in that document.

The present invention is broadly directed to methods of at least inhibiting growth of anaerobic microorganisms using BLIS-producing *S. salivarius* strains or compositions comprising same, or at least provides the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, in one aspect the invention provides a method for at least inhibiting the growth of anaerobic bacteria sensitive to BLIS-producing *S. salivarius,* the method comprising contacting the sensitive bacteria with an inhibitory effective amount of a BLIS-producing *S. salivarius*, or an extract, or a composition or formulation containing same.

In a further aspect, the invention provides a method of prophylactic or therapeutic treatment of halitosis in an individual in need thereof, the method comprising administering to said individual a BLIS-producing *S. salivarius*, extract, composition or formulation in an amount effective to at least inhibit growth of anaerobic bacteria or in an amount to allow effective colonisation in the oral cavity of the individual.

Preferably the *S. salivarius* are *Salivaricin B* producers.

Preferably, the anaerobic bacteria are black pigmented species, *Eubacterium* and/or *Micromonas*, especially *Prevotella* species, *Eubacterium saburreum* and *Micromonas micros*.

In a further aspect, the invention provides a method of controlling the incidence and severity of halitosis comprising introducing into the oral cavity of an individual susceptible to halitosis, a halitosis controlling amount of a BLIS-producing *S. salivarius*, extract, composition or formulation.

In one embodiment the halitosis is caused by black pigmented species, *Eubacterium* or *Micromonas*, particularly *Prevotella* species, *Eubacterium saburreum* and *Micromonas micros*.

Preferably, *S. salivarius* is administered as part of a lozenge, spray or other drug delivery device, confectionary, food, drink or nutraceutical.

The methods of the invention preferably include the preliminary step of pre-treating the individual to at least reduce the oral microflora already present.

The invention also relates to the use of BLIS-producing *S. salivarius*, extracts, compositions or formulations in the methods discussed above. Particlarly, to the use of the *S. salivarius* in the preparation of medicaments for use in treating halitosis.

In another aspect, the invention also relates to the use of BLIS-producing *S. salivarius* strains and active extracts in the methods discussed above for inhibiting, controlling, preventing or treating halitosis caused at least in part by *Prevotella* species, *Eubacterium saburreum* and *Micromonas micros*.

Although the invention is broadly as described above, it will be appreciated by those persons skilled in the art that the invention is not limited thereto but also includes embodiments of which the following description gives examples. In particular, the invention will be described in relation to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Example of inhibitory effect of S. salivarius K12 on black-pigmented bacteria from saliva sample.

Figure 2. DGGE amplicons from bacterial DNA in subjects saliva samples. A) Subjects prior to treatment. B) Subjects with decrease in VSC's at day 14. C) Subjects without decrease in VSC's at day 14.

Figure 3. DGGE of bacterial amplicons from two case subjects.

Figure 4. DGGE of bacterial amplicons utilizing Streptococcus-specific PCR primers from two case subjects.

Figure 5A. VSC levels of mouth air from two case subjects (4 and 12) over 28 days and after treatment.

Figure 5B. Detection of BLIS activity of Streptococcus salivarius isolates (%) from case subjects over time by sensitive indicator microorganism Micrococcus leuteus (I1, sensitive to SAL A and B).

Figure 5C. Bacterial counts of saliva from subject 4.

Figure 5D. Bacterial counts of saliva from subject 12.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is directed in a first aspect to a method for at least inhibiting the growth of anaerobic bacteria sensitive to BLIS-producing *S. salivarius*. The method comprises contacting the sensitive bacteria with an inhibitory effective amount of a BLIS-producing *S. salivarius*, or an extract, or a composition or formulation containing same.

In another embodiment the invention relates to methods of prophylactially or therapeutically treating halitosis, and to methods of controlling the incidence and severity of halitosis as set out above.

Preferably, the *S. salivarius* strains for use in the invention are *Salivaricin B* producers with activity against anaerobic bacteria, particularly black pigmented species such as *Prevotella*, *Eubacterium saburreum* and/or *Micromonas micros*. BLIS-producing strains with activity against anaerobic bacteria include K12, and K30 both deposited with Deutche Sammlung von Mikroorganismen Und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124, Braunschweig, Germany on 8 October 1999, and 8 October 1999, and assigned Accession Nos. DSM 13084 and 13085 respectively.

Strain Sal 20P3 was deposited at the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, New South Wales, Australia in July 1992 under Accession No. AGAL 92/32401.

While *Salivaricin A* producer 20P3 has activity against *Micromonas*, *Salivaricin B* producers K12 and K30 have a broader range of activity against black pigmented species, *Eubacterium* and *Micromonas* at least.

As noted above black pigmented species, *Eubacterium* and *Micromonas* are considered causative agents in halitosis. While these BLIS-producing strains of *S. salivarius* are known to be active against gram-positive aerobic bacteria, their activity against anaerobic bacteria, such as black pigmented species, *Eubacterium* and *Micromonas* in particular, is unexpected. All the more so because BLIS-producing organisms are typically known to act against more closely related species.

These BLIS-producing *S. salivarius* are therefore useful as anaerobic antibacterial agents *per se* as well as therapeutically. In this context, "therapeutic" includes prophylactic treatment. Therapeutic uses include the treatment or prevention of anaerobic microbial infections, especially *Eubacterium* and *Micromonas* infections, and infections by black pigmented species. The *S. salivarius* are particularly suitable for use against *Prevotella* species, *Eubacterium saburreum* and *Micromonas micros*. Conditions amenable to treatment with the *salivarius* include halitosis and bad breath.

Extracts obtainable from the BLIS-producing *salivarius* strains are also useful in the invention. These active extracts may similarly be used in therapeutic formulations and methods. Extracts can be obtained using known art protocols, conveniently by cell culture and centrifugation. Extracts include the antibiotics salivaricin A and salivaricin B in isolated or pure form.

A "therapeutic formulation" is a formulation appropriate for administration of an *S. salivarius* strain or extract herein, to an individual in need of same, particularly a halitosis-susceptible individual. In general, therapeutic formulations are composed of an *S. salivarius* strain or extract discussed above and an acceptable carrier, diluent and/or excipient.

An "acceptable carrier, diluent and/or excipient" means a vehicle for delivery of a *S. salivarius* strain or extract, to the individual, in which the vehicle is compatible with bacterial cell viability, or activity of the extract. Acceptable carriers suitable for use in the administration of viable *S. salivarius* strains and extracts are well known to those skilled in the art. Suitable carriers are generally inert and can be either solid or liquid.

In one embodiment, the carrier is a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers suitable for use with the *S. salivarius* strains herein include, but are not limited to, water, buffered saline solutions (e.g., phosphate-buffered saline), pharmaceutically acceptable culture media (e.g. BACa, TSBCaYE agar), or other solutions which maintain the viability of the bacterium. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. A variety of pharmaceutically acceptable carriers suitable for oral administration of viable or lyophilized bacteria are well known in the art (see, for example, *Remington's Pharmaceutical Sciences*, 18th ed., Gennaro, ed., 1990, Mack Publishing Co., Easton, Pa., incorporated herein by reference; and the pharmaceutical composition LACTINEX™, a commercially available formulation for oral administration of viable lactobacilli). Suitable solid carriers known in the art include, for example, magnesium carbonate; magnesium stearate; celluloses; talc; sugars such as fructose, sucrose, mannitol, lactose; starches; flours; oligosaccharides and skim milk, and similar edible powders, but are not limited thereto. Carriers for administration of extracts are similarly well known.

Typical diluents, by way of example, are: starches; lactose; mannitol; kaolin; calcium phosphate or sulphate; inorganic salts such as sodium chloride; and powdered sugars or celluloses.

The compositions may also include excipients such as tableting aids; resins; fillers; binders; lubricants; solvents; glidants; disintegrants; preservatives; buffers; flavourings; colourings; sweeteners; and fragrances as appropriate. A preferred excipient for tablet flowability and compactability is ProSolv™ (Penwest, NY, USA). A preferred sweetener is isomalt.

Typical binders include starch; gelatin; sugars such as lactose, fructose, and glucose; and the like. Natural and synthetic gums are also convenient, including acacia; alginates; locust bean gum; methylcellulose; polyvinylpyrrolidine tragacanth; Xanthan gum: and the like. Polyethylene glycol; ethyl cellulose; and waxes can also serve as binders. A currently preferred binder is Emdex™ (Penwest, NY, USA).

Lubricants to prevent sticking to the die during formation include slippery solids such as talc, silica, magnesium and calcium stearate, polyethylene glycol, stearic acid and hydrogenated vegetable oils.

Disintegrators are substances which swell when wetted to break up the lozenge and release the *S. salivarius* or extract. The disintegrators include starches; clays; celluloses; algins and gums; more particularly corn and potato starches; methylcellulose; agar; bentonite; wood cellulose; cation exchange resins; alginic acid; guar gum; citrus pulp; carboxymethylcellulose; powdered sponge; and sodium lauryl sulfate.

The *S. salivarius* strains or extracts herein can be formulated in any of a variety of compositions suitable for oral administration. For example, the *S. salivarius* strains can be formulated for administration as a lyophil or cell paste prepared from a *S. salivarius* culture, or can be directly administered to the oral cavity. The strain or extract can also be administered in the form of a mouthwash, mouth rinse, toothpaste, mouthspray, gargle, capsule, lozenge, syrup, floss, chewing gum, or chewable tablet but the forms are not limited thereto.

Therapeutic formulations may include food, confectionary or drink. In one embodiment, the foodstuff or drink is a dairy product-based food or drink including by way of example,

yoghurt, cheese, milk, milk power, milk biscuits, and flavoured milks. In the case of confectionary, the formulation can be a chewing gum such as described in WO 00/05972. One preferred formulation employs freeze dried *S. salivarius* strains herein, in milk powder formulations in a manner similar to that previously reported for the preparation of Bifidus Milk Powder (Nagawa et al. (1988); J. Dairy Sci. 71:1777-1782).

One orally administrable formulation of *S. salivarius* is a blend of freeze dried *S. salivarius* strains with skim milk powder or the like which has been flavoured to enhance palatability.

Presently preferred orally administrable formulations of *S. salivarius*, or extracts herein are lozenges, chewable tablets, or capsules. Lozenges are particularly preferred. A suckable lozenge preferably comprises an *S. salivarius* strain or extract, isomalt and emdex. The lozenge may be prepared by direct compression, wet granulation, or dry granulation. The lozenges may be coated according to well known pharmaceutical practice.

The therapeutic formulation can additionally contain nutrients to maintain the viability of the bacterium in the formulation. As noted above, the formulation can also contain flavouring agents, colouring agents, fragrances, or other compounds which increase the palatability of the composition and/or enhance patient compliance without compromising the effectiveness of the formulation. Methods for preparation of formulations for oral administration are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th ed., supra, incorporated herein by reference).

For general antimicrobial use, formulations may also be produced for other methods of administration including topically administrable formulations but not limited thereto.

The formulations and compositions may further comprise one or more secondary antibacterial agents. These secondary agents may, for example, be antibiotics, or other antibacterial agent or antibacterial producing microorganisms. Preferably, the secondary antibacterial agent is a BLIS or BLIS producing microorganism. The BLIS may be one or more of salivaricin A, A_1, A_2 and B.

Secondary agents useful in such a composition may be odour masking or neutralising agents such as peppermint, chlorine dioxide, zinc, baking soda or other agents with a similar purpose.

Other ingredients useful in such a composition are anticariogenic agents, for example Xylitol, fluoride, and calcium.

Further ingredients useful in such a composition are agents that selectively enhance growth of desirable bacteria over non desirable organisms. These agents may, for example, be oligosaccharides such as Nutriose® FB.

In the treatment of halitosis, *S. salivarius* strains or extracts can be administered to any individual susceptible to halitosis, usually an individual in which black pigmented species, *Eubacterium* and/or *Micromonas* colonises the oral cavity such that the halitosis is caused at least in part by black pigmented species, *Eubacterium* and *Micromonas*.

The term "individual" as used herein includes humans, horses, dogs, cats, pigs, sheep, cattle, goats but is not limited thereto. Preferably, the individual is a human. The *S. salivarius* strains can be administered to the individual at any age, e.g. childhood, adolescence, or adulthood.

S. salivarius herein can be orally administered in a variety of ways. For example, in the form of compositions or formulations discussed above, or as suspensions, sustained release formulas (e.g. an oral implant containing the *S. salivarius* strain) or lyophil powders. The *S. salivarius* strains can also be administered by direct application of a lyophil, culture, or cell paste to the oral cavity of the individual. Any mode of administration is suitable as long as the therapeutic formulation is applied to the oral cavity. In one embodiment, the *S. salivarius* or extracts are administered by applying directly to the tongue of the individual, e.g. by brushing.

In general, the amount of *S. salivarius* administered to the individual will be an amount effective for replacement of halitosis-causing anaerobic bacteria strains, or at least black pigmented species, *Eubacterium* and/or *Micromonas* in the oral cavity of the host. "An amount effective for replacement of halitosis-causing anaerobic bacterial strains or at least black pigmented species, *Eubacterium* and/or *Micromonas* in the oral cavity of the host" means an amount effective for oral cavity colonisation by the *S. salivarius* strain, and significant reduction of the resident halitosis-causing anaerobic bacteria (e.g. by competition

between the bacteria for nutrients and/or by the production of BLIS by the *S. salivarius* strain).

The term "unit dose" when used in reference to a therapeutic formulation herein refers to physically discrete units suitable as unitary dosage for the individual, each unit containing a predetermined quantity of active material (viable *S. salivarius* or active extract thereof) calculated to produce the desired therapeutic effect in association with the required diluent, carrier, or excipient.

Specific dosages can vary widely according to various individual variables including size, weight, age, disease severity (e.g. the tenacity and/or number of halitosis-causing resident bacteria) and responsiveness to therapy (e.g. the susceptibility of the individual's oral cavity to colonisation). Methods for determining the appropriate route of administration and dosage may be determined by the consumer as they deem appropriate, or on a case-by-case basis by an attending dentist or other clinician. Such determinations are routine to one of ordinary skill in the art (see for example, *Remington's Pharmaceutical Sciences*, 8th ed., Gennaro, ed., Mack Publishing Company, Easton, Pa., 1990).

In general, the number of *S. salivarius* administered to the individual will range from about 10^2 to 10^{15} bacteria, preferably from about 10^3 to 10^{14} bacteria, more preferably from about 10^5 to 10^{12} bacteria, normally about 10^9 to 10^{10} colony forming units (CFU) per dose. One formulation employs 3.8×10^9 CFU/lozenge.

Multiple doses of the *S. salivarius* strain can be administered to achieve oral cavity colonisation and replacement of the resident, halitosis-causing strains, particularly black pigmented species, *Eubacterium* and/or *Micromonas* of the individual. The *S. salivarius* strain or extract may need to be administered to the patient once only or more usually repeatedly. Repeat treatments may be once a month, once a week, once a day, twice a day, or as may otherwise be required. Conveniently, the administration may be effected as part of the patient's routine dental care, e.g. as a component of a lozenge, gum, toothpaste, floss, or mouthwash.

To facilitate colonisation, in one embodiment the treatment method of the invention includes a preliminary step of pre-treating the individual to at least reduce the normal microflora present in the oral cavity, including halitosis causing organisms. This pre-treatment

comprises the step of administering an antimicrobial agent such as chlorhexidine, chlorine dioxide, triclosan, lactoperoxidase, green tea, or pineapple juice (freeze dried), but not limited thereto. Where chlorine dioxide is used, it is preferably mixed with water or desirably fruit juice prior to administration. The pre-treatment may also include physical removal methods such as brushing or scaping, or may follow a prescribed course of antibiotics such as tetracyclines, penicillin, erythromycin, metronidazole, or amoxycillin administered to said individual. *S. salivarius*, extracts, formulations or compositions containing same are then administered to the depopulated environment to repopulate same.

A currently preferred treatment protocol for halitosis comprises pre-treatment by scraping the tongue brushing teeth and tongue with antibacterial toothpaste (e.g. Perioguard, 2% chlorhexidine); gargling or rinsing with chlorhexidine (e.g. a rinse with 0.2% chlorhexidine); then taking a lozenge. In an alternate preferred protocol for halitosis the pre-treatment comprises scraping the tongue and optionally brushing teeth and tongue with a non-chlorhexidene containing toothpaste; gargling or rinsing with chlorine dioxide; then taking a lozenge. In each case, a lozenge is administered 1-4 hours, preferably 2 hours after the pre-treatment. This is followed by administration of a further 2-5, preferably 3 lozenges through the day at intervals of 1-4 hours, preferably every 2 hours. This protocol is followed for 2-4 days to facilitate colonisation. Usually, in this period the teeth and tongue are brushed and the gargle or rinse continued. However, the brushing with chlorhexidine toothpaste is discontinued. For maintenance purposes 1, 2, or 3 lozenges, usually 1 to 2 lozenges are taken each day following ordinary tooth brushing. The regime is continued for as long as required.

Successful colonisation of the individual's oral cavity by the *S. salivarius* strain can be established by culturing the bacteria of the individual's oral cavity, and identifying the *S. salivarius* strain by, for example, BLIS production or other methods well known in the art for bacterial strain identification.

The methods and uses of the invention may further comprise the use of one or more secondary antibacterial agents as discussed above.

Where the term comprise, comprises, comprised or comprising are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

Various aspects of the invention will now be illustrated in a non-limiting way by reference to the following experimental section.

EXPERIMENTAL

Deferred Antagonism Test of Anti-bacterial Activity

The spectrum of inhibitory activity of *Streptococcus salivarius* K12 was established by use of a deferred antagonism test, essentially as described by Tagg and Bannister (J. Med. Microbiol. 1979;12:397). In brief, a 1-cm wide diametric streak culture of K12 (producer strain) was inoculated onto blood agar-calcium medium (Columbia agar base plates, 5% human blood, 0.1% $CaCO_3$ [BDH]). Following incubation in a 5% CO_2 atmosphere, for 24 hours at 37°C, the macroscopic cell growth was removed with a glass slide and residual cells on the agar surface were killed by exposure to chloroform vapours for 30 minutes. The agar surface was then aired for 30 minutes. Indicator strains implicated in halitosis which had been grown for 48 hour on blood agar-calcium plates, were suspended in Todd Hewitt broth and used to inoculate at 90-degree angles across the line of the original streak culture with the use of sterile cotton swabs, under anaerobic conditions. After incubation for 48 hours in an anaerobic environment at 37°C the extent of inhibition of each indicator strain was recorded. The scoring system for the measurement of inhibition was the following: a negative sign (-) denoted no inhibition of the indicator microorganism; a positive symbol (+) indicated that there was some inhibition on the plate where the producer microorganism had grown, but not exclusively over that entire region; two positive symbols (++) denoted that the indicator strain was inhibited where the producer strain was grown; when three positive symbols (+++) were used, growth of the indicator strain was at least 5 mm away from where the producer strain had grown; and four positive symbols (++++) represented inhibition of the indicator strains beyond 5 mm of growth. The results are shown in Table 1 below.

Inhibition of black-pigmented bacteria in saliva by S. salivarius strains

To test the ability of *S. salivarius* against black-pigmented microorganisms which have been implicated in halitosis, *S. salivarius* strains K12 (SAL A and B producer), NR (SAL B producer), 20P3 (SAL A producer) and MU-Neg (non-producer) were swabbed from master plates onto the entire half of several pH-buffered blood plates (Columbia agar base plates, 5% human blood, 0.1% $CaCO_3$ [BDH]). Plates were incubated for 18 hours at 37°C under 5 % CO_2 conditions. Bacterial growth by removed using cotton-tipped swabs and chloroform vapours were used to kill any remaining bacteria. Fifteen millilitres of vancomycin blood agar

(per L; 30 g trypticase soy broth [BBL], 15 g agar [BBL] and after autoclaving, 50 ml of defibrinated blood and 10 ml of filter-sterilized vancomycin stock solution [0.014g in 10 ml H_2O]) was overlaid on plates and allowed to set. Fresh saliva-samples from two 'normal' subjects were diluted in sterile saline and 50 ul aliquots were immediately spiral plated on to the prepared agar plates. Plates were incubated under anaerobic conditions at 37°C for 48-72 hours depending on growth. Both sectors of the plate were counted.

Subjects, treatment, probiotic instillation and sample collection

Sixty-five subjects were screened for volatile sulphur compound (VSC) breath readings by halimeter (Interscan Corp., Chatsworth, CA). VSC readings in persons with normal breath are typically in the range of 80-150 ppb. At levels of 200-300 ppb oral malodor is noticeable by an observer standing close to the patient. At 350-400 ppb, the odour is noticeable by an observer standing several feet away from the patient. Those subjects with breath scores of greater than 200 ppb on two separate visits were were recruited for the study (n=13). Five subjects with 'normal' VSC levels were also recruited from the laboratory. Readings are given in Table A below.

Table A. Subject readings of the normal group

Demographics			Results of sampling		
Subject (n=7)	Age	Sex	VSC (ppb)	BANA	Saliva organoleptic
28	25	F	135	+	2
30	76	M	179	-	2
70	31	M	84	+/-	0
71	20	M	79	+/-	2
72	35	F	55	-	1
73	25	M	170	-	1
74	31	M	98	-	1
Mean (SD)					

Each signed an Informed Consent under a protocol approved by the Otago Ethics Committee. Subject's upon their second positive visit undertook a mechanical and chemical treatment of their mouths at the laboratory. This consisted of teeth and tongue brushing (2 minutes with Colgate Total [0.3% w/v triclosan]), tongue scrapping for 30 seconds, brushing teeth and tongue with a CHX gel (Perioguard 2.0% CHX mouth gel, 2 minutes) and followed by a 30 second CHX rinse (0.2% CHX, 10% ethanol alcohol). This was followed at intervals by the sucking of a lozenge (commencing 2 hours after treatment and typically 2 hours apart, 4 times daily), each containing >1 x 10^9 colony forming units of streptomycin-marked *S. salivarius*

K12. On days 2 and 3, subjects brushed their teeth and tongue in the morning with Colgate Total, used a 30 second CHX rinse (0.2% CHX, 10% ethanol alcohol) and used K12 lozenges as per the first day. The subjects then ceased with the chlorhexidine treatment and took just two lozenges per day on days 4 to 14 after conducting their normal oral care regimen, morning and night.

At each pre-treatment visit and at one and two weeks after treatment initiation the subjects were tested for VSC levels and both saliva samples and tongue swabs were taken (prior to taking morning lozenge). During the study two subjects were sampled more frequently and kept on two lozenges for a period of 28 days. These subjects provided additional samples at day 4, 11, 21, 28 and two weeks post ceasing K12 administration. All measurements were taken in the morning prior to the subjects eating, drinking smoking or using any oral care.

After the study 4 subjects who responded positively to the treatment were followed up six weeks later. To determine the effect of the chlorhexidine treatment on breath scores subjects were asked to repeat the study using the same 3-day CHX regimen, but with no *S. salivarius* probiotic. As for the study subjects were seen on the seventh day for measurements and samples.

Saliva analysis

Fresh saliva samples and tongue swabs were tested for *N*-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis (BANAMet ILC, Ann Arbor, MI.), since positive scores by the BANA test have previously been associated with a component of oral malodor (1, 3). This was conducted according to the manufacturers' directions. Saliva was also subjected to an organoleptic scoring system, conducted simultaneously on all samples from thawed frozen samples. Briefly, 300 ul of thawed saliva sample from each subject and time point were incubated at 37°C for 10 minutes in a closed eppendorf. After incubation, the vessel was opened at approximately 4 cm from the examiners nose for sampling. Samples were graded under the same criteria used to assess breath air from halitosis sufferers (7), using a scale of 0-5, with 0 meaning no odour detected and 5 most severe.

Cultural analysis

Fresh saliva samples were immediately diluted ten-fold in sterile phosphate-buffered saline (PBS, pH 7.5). Fifty-micro litre aliquots were spiral plated onto pH-buffered blood plates (as previously described), Mitis-salivarius (Difco, *S. salivarius* selective) and K12 selective

Mitis-salivarius media containing streptomycin (100 ug/ ml, Sigma, Mo). Plates were incubated in a 5% CO_2 atmosphere, for 24 hours at 37°C.

Detection of bacteriocin-like inhibitory substances (BLIS)

pH-buffered blood plates (as previously described) were inoculated across in a lawn with a suspension of *Micrococcus leuteus* (I1), which is sensitive to production of the bacteriocins including salivarcin A and B. For each of the two case subjects that were studied in detail, 50 – 100 *S. salivarius* colonies picked from the Mitis-salivarius–specific media used to enumerate and were stabbed into the pre inoculated pH-buffered blood plates. Plates were incubated in a 5% CO_2 atmosphere, for 24 hours at 37°C. Those stabs with a zone of inhibition considered to be positive were counted and presented as a percentage of the overall number.

Extraction of bacterial DNA from saliva for PCR

Saliva samples (400 ul) was mixed to a volume of 1 ml with phosphate-buffered saline (PBS, pH 7.5). Cells were pelleted by centrifugation (10 000 × *g*, 5 minutes) and total DNA was extracted using Instagene Matrix (Bio-Rad Laboratories, Hercules, Ca), according to the manufacturers' instructions and stored at –20°C. PCR reactions were carried out in 0.2 ml tubes in a thermocycler (Eppendorf Mastercycler, Germany). The HDA eubacterial and *Streptococcus*-specific PCR primers and amplification conditions have been previously described and tested on clinical samples (2, 4, 6).

DGGE, DNA fragment excision from gels, re-amplification and sequencing

Preparation of DGGE gel gradients and electrophoresis were carried out according to the manufacturers' guidelines for the CBS Scientific DGGE apparatus (Del Mar, CA). A 100 % solution was taken as a mixture of 7 M urea and 40 % formamide. The concentration of polyacrylamide, denaturant and TAE was 8 %, 40 - 55 % and 1 ×, respectively. Gels were run at 70 V for 15.5 hours. Fragments of interest were excised from DGGE gels by sterile scalpel, washed once in 1 × PCR buffer and incubated in 20 µl of the same buffer overnight at 4°C. Five-microlitres of the buffer solution was used as template for PCR. Re-amplification was conducted using the same primers as described, but without the "GC-clamp". Sequences of the re-amplified fragments were determined by the dideoxy chain termination method (Sequencing Facility, Center for Gene Research, University of Otago). Analysis of the partial 16S rRNA sequences was conducted using Genbank and the BLAST algorithm. Identities of isolates were determined on the basis of the highest score.

Results

Inhibitory effect

The testing of *Streptococcus salivarius* strains which produced SAL A and B, SAL A only, SAL B only or a non producer against some organisms implicated in halitosis showed that only the gram-positive bacteria were affected when tested by deferred antagonism testing (Table 1.).

Table 1. Sensitivities of selected microoganisms implicated in halitosis to various *S. salivarius* strains

Control strain	Control strain	Inhibition of growth of organisms implicated in halitosis							
S. salivarius strain	Bacteriocin/s produced	*Micrococcus luteus* I1.	*S. anginosis* I3.	*Eubacterium saburreum* ATCC 33271	*Micromonas micros* ATCC 33270	Porphymonas gingivalis ATCC 33277	*Porphymonas gingivalis* W50	*Prevotella intermedia* ATCC 25611
K12	Salivaricin A Salivaricin B	++++	++++	++++	++++	-	-	-
NR	Salivaricin B	++++	++++	++	++++	-	-	-
20P3	Salivaricin A	++++	-	++	-	-	-	-
MU Neg.	Non producer	-	-	-	-	-	-	-

Additionally, the maximum amount of inhibition of the gram-positives was obtained by K12, which produces both bacteriocins A and B. The next potent *S. salivarius* was NR, a SAL B only producer, which had good activity against the *Micromonas micros* strain but reduced activity against the *Eubacterium saburreum* strain compared to K12. The non bacteriocin-producing strain (MU Neg.), did not inhibit any of the strains tested.

Effect against organisms in saliva

In both subjects the largest reduction (8 and 37% of respective counts) of black-pigmented microorganisms appeared on the control half of the agar plate where K12 (SAL A, B producer) had grown (Table 2., Fig1.).

Table 2. Effect of *S. salivarius* strains on black-pigmented anaerobe numbers in saliva

Subject	*Streptococcus salivarius* strain and numbers*							
	K-12	Control-half	NR	Control-half	20P3	Control-half	MU Neg	Control-half
A	1.4×10^5	1.7×10^6	1.4×10^6	3.0×10^6	2.0×10^6	3.5×10^6	1.8×10^6	2.2×10^6
B	3.0×10^6	7.9×10^6	4.1×10^6	6.9×10^6	3.1×10^6	6.6×10^6	5.4×10^6	2.6×10^6

*CFU/ml of black-pigmented colony morphology.

There was also reduction in the plates, which had SAL A or B only producers grown, but little on the control plate which had a non-producing strain. Several colonies were picked from the control half of the plates, DNA extracted, amplified by PCR and DNA sequenced. Sequencing results indicated that *Prevotella* sp. oral clone BE073 (closely related to *Prevotella melaninogenica*) 99% 152bp (AF385551) had the highest homology.

Clinical data

All subjects completed the two-week study. VSC readings decreased greater than 100 ppb in 11/13 subjects when measured at 7 days and 8 of those subjects maintained lower than pre-treatment levels when measured at day 14 (Table 3.).

Table 3. Subject readings at baseline and after treatment

Demographics			Baseline						Treatment					
			Initial Screen			Second Visit			Day 7			Day 14		
Subject (n=13)	Age	Sex	VSC (ppb)	BANA	Saliva organoleptic	VSC (ppb)	BANA	Saliva organoleptic	VSC (ppb)	BANA	Saliva organoleptic	VSC (ppb)	BANA	Saliva organoleptic
4	18	F	386	+/-	3	504	-	ND	175	+/-	2	222	-	2
5	20	M	292	ND	ND	394	ND	ND	159	-	1	97	-	2
12	18	M	404	+	3	434	+/-	ND	59	+/-	1	364	-	2
27	25	F	324	-	3	514	-	3	128	-	1	145	-	1
35	39	M	320	-	4	200	ND	ND	631	-	5	413	+/-	4
38	69	M	363	+/-	3	290	+	3	197	+/-	2	358	-	3
46	47	M	229	+	3	327	+	4	75	+/-	2	266	+/-	4
47	64	F	307	+	3	443	+	3	126	+/-	2	183	+/-	3
52	68	M	486	+	4	312	+/-	3	109	+/-	3	56	-	2
58	68	M	408	-	3	213	+/-	3	154	+/-	2	122	-	1
59	33	F	254	+	3	206	+/-	2	304	+/-	1	303	-	2
60	41	M	433	+/-	4	381	-	3	287	-	3	244	-	3
64	43	M	286	+	3	210	+/-	2	132	-	2	135	-	1
Mean (SD)	42.54 (19.59)	F=4 M=9	345.54 (75.06)		3.25 (0.45)	340.62 (113.74)		2.88 (0.60)	195.08 (149.26)		2.077 (1.12)	223.69 (112.65)		2.307 (1.03)

+/-=weak positive.
ND=not determined.

The BANA readings were also lower in most subjects post treatment, where no strong positives were detected, whereas several were detected prior. Changes in the organoleptic readings also generally correlated with the other clinical parameters that were measured.

Four subjects who had maintained lower breath readings and parameters during the K12 treatment were recalled six weeks later for monitoring the effect of the chlorhexidine alone. One subject was excluded as their VSC breath levels had not returned to greater than 200 ppb and had been taking the commercially available K12 product. There was no decrease in the VSC, organoleptic or BANA readings of the other three subjects after treatment with CHX only (Table 4.).

Table 4. Subject readings of three recalled subjects before and after chlorhexidine

Subject	Mean VSC (ppb) change in CHX & K12 trial	Baseline			Post CHX treatment			VSC (ppb) change after CHX only
		VSC (ppb)	BANA	Saliva organoleptic	VSC (ppb)	BANA	Saliva organoleptic	
27	-282.5	532	+/-	2	527	+	2	-5
47	-220.5	298	+/-	3	244	+/-	3	-54
60	-141.5	216	+/-	3	274	+/-	2	+58

Culture data

On average, there was no significant change in total bacterial counts of the subjects either before or after treatment, however, there was a slight though non-significant increase in S. salivarius numbers (Table 5.).

Table 5. Colony forming units per ml on various media at baseline and after treatment.

Media	Baseline		Treatment		Significance
	Initial Screen	Second Visit	Day 7	Day 14	
Columbia Blood & Calcium CO_2	8.7×10^8 (1.4×10^9)	2.4×10^8 (2.7×10^8)	3.3×10^8 (4.8×10^8)	$2.3e^8$ (3.7×10^8)	NS
Mitis salivarius CO_2	4.4×10^7 (6.8×10^7)	4.5×10^7 (6.2×10^7)	6.1×10^7 (7.8×10^7)	6.1×10^7 (6.6×10^7)	NS
Mitis salivarus-streptomycin CO_2	ND	ND	1.8×10^7 (4.9×10^7)	5.8×10^7 (1.4×10^7)	ND

NS=Not significant $p>0.5$.
ND=Not determined.

There was also no significant difference between the total cell counts (2.82×10^8 vs 1.58×10^8 CFU/ ml, $p>0.5$) or S. salivarius cell counts (7.21×10^7 vs 4.35×10^7 CFU/ ml, $p>0.5$) for those subjects who had maintained low readings successfully or those who had reverted to readings similar to pre-treatment. However, when S. salivarius was examined as a percentage of the total population in each individual, 11 out of 13 subjects increased the ratio of S. salivarius as the total proportion when comparing the results prior to those after treatment (data not shown).

Analysis of the cultural data from the two case subjects (subjects 4 and 12) showed that the highest *S. salivarius* to total counts was at the day 7 time point when the species made up to 70% of the total population for subject 4 and above 20% for subject 12 (Fig 4.). This time point also correlated to the lowest VSC readings for both subjects for the entire study. The proportion of *S. salivarius* isolates that produced bacteriocin-like inhibitory substances (BLIS) varied between the case subjects in the study. Both subjects had the highest proportion of BLIS producers at day 7; however, subject 4 always had less than 10% of their total *S. salivarius* count that produced BLIS, whereas, Subject 12 at times was almost entirely occupied by these *S. salivarius* subsets.

DGGE analysis

Samples from subjects prior to treatment generally had quite a similar profile, especially with the reoccurrence of the dominant fragments between samples. This was also the case when compared to saliva from 5 normal control subjects (data not shown). Fragment sequence similarities are listed in Table 6.

Table 6. BLAST analysis of bacterial V2-V3 16S rRNA sequences of excised fragments from DGGE gels.

Fragment in gel	Most closely related bacterial sequence and nearest species match	% Identity and bp searched	Accession number
1	*Prevotella* sp. oral clone FM005	99% (151 bp)	AF432133
	Prevotella veroralis ATCC 33779	99% (150 bp)	L16473
2	*Prevotella* sp. oral clone GI030	97% (154 bp)	AY349395
	Prevotella melaninogenica ATCC 25845	97% (154 bp)	L16469
3	Uncultured *Streptococcus* sp.	98% (172 bp)	AY307987
	Veillonella dispar	98% (172 bp)	AF439639
4	*Prevotella* sp. oral clone FM005	99% (151 bp)	AF432133
	Prevotella veroralis ATCC 33779	99% (150 bp)	L16473
5	*Prevotella* sp. oral clone GI030	100% (152 bp)	AY349395
	Prevotella melaninogenica ATCC 25845	100% (152 bp)	L16469
6	Uncultured *Streptococcus* sp.	95% (172 bp)	AY307987
	Veillonella dispar	95% (172 bp)	AF439639
7	unidentified oral bacterium AP60-60	99% (152 bp)	AB028410
	Neisseria pharyngis NCTC 4590	98% (152 bp)	X74893
8	*Prevotella pallens*	98% (167 bp)	Y13106
9	*Prevotella* sp. oral clone FM005	99% (152 bp)	AF432133
	Prevotella veroralis ATCC 33779	99% (152 bp)	L16473.
10	*Prevotella melaninogenica* ATCC 25845	100% (152 bp)	AY323525
11	Uncultured *Streptococcus* sp.	99% (173 bp)	AY307987
	Veillonella dispar	99% (171 bp)	AF439639

Many profiles changed after treatment (Fig. 2). This is highlighted especially with the two case subjects who were studied in greater detail, taking on more time points (Fig. 3). Comparing the subjects who maintained low VSC and other readings at day 14 compared to those that did not, typically had similar profiles. However, there was at least one difference, in that those that maintained low readings typically had a fragment appearing where the DNA fragment for *S. salivarius* K12 had migrated to, probably indicating the presence of this or a similar microorganism.

DGGE profiles of the two case subjects where tracked for twenty-eight days and had some interesting changes. Subject 4 did not show immediate change after the chlorhexidine and *S. salivarius* K12 treatment, other than a fragment parallel to that of K12 appeared. The

microbiota of this subject as demonstrated by DGGE appeared to change dramatically when sampled on days 11 and 14. After this the DGGE profile returns to one which is similar to that detected earlier. Subject 12's DGGE profile changes immediately after the sample after treatment commences. A fragment, which migrated parallel to that of *S. salivarius* K12 appears to be amongst the predominant bands. As with subject 4, there appears to be a major change in the composition of the microbiota at days 11 and 14. By day 21, 28 and post sample the microbiota appears to have changed again, but with similarities to that of several previous samples.

Streptococcus-specific DGGE

These PCR products show that subject 4 has a gradual increase in fragment intensity where *S. salivarius* would be expected to migrate to until the peak at day 14. The fragment disappears at day 21 and reappears at day 28 and at the post test sample. Subject 12 has intense fragments where *S. salivarius* would be expected to migrate to on days 4 and 7 after commencing treatment. This fragment is detectable at days 11 and 14, but disappears at day 21, but returns at the post sample.

Discussion

Streptococcus salivarius K12 was detectable in each subject's saliva (median of 8.2×10^5 CFU/ ml) for both post samples for at least 12 hours, as study participants were sampled in the morning after receiving their last dose the night previous.

When examining the percentage of *S. salivarius* as a proportion of the total bacterial composition, 85% of the subjects had an increase in the proportion of this microorganism as part of the total count, suggesting that it had become more common in the oral cavity.

The VSC levels of eight subjects were significantly lower when tested one and two weeks after commencing treatment, three subjects had lower readings only after one week and two subjects maintained high levels throughout the study. Reduction of BANA activity and changes in the PCR-DGGE profiles occurred in most subjects following treatment. In deferred antagonism studies, *S. salivarius* K12 inhibited gram positive bacterial species implicated in halitosis, and significantly inhibited black-pigmented colony types in saliva samples.

Based on these studies, the replacement of bacteria implicated in halitosis with bacteriocin-producing commensal bacterium, particularly *S. salivarius* K12, appears to provide an alternative therapy for the long term reduction of halitosis.

INDUSTRIAL APPLICATION

BLIS-producing *S. salivarius* strains, particularly *Salivaricin B* producing strains are active against a number of microorganisms implicated in halitosis (Tables 1 and 2). The strains and related active extracts herein therefore have application in methods of therapeutically treating individuals against the harmful effects at least of *Eubacterium* and *Micromonas* infection, as well as black-pigmented colony types, especially in the oral cavity. These methods include treatment of halitosis in which these organisms are the primary causative agents. The *S. salivarius* extracts and compositions of the invention also have application in the treatment of sore throats.

It will be appreciated that the above description is provided by way of example only and that variations in both the materials and techniques used which are known to those persons skilled in the art are contemplated.

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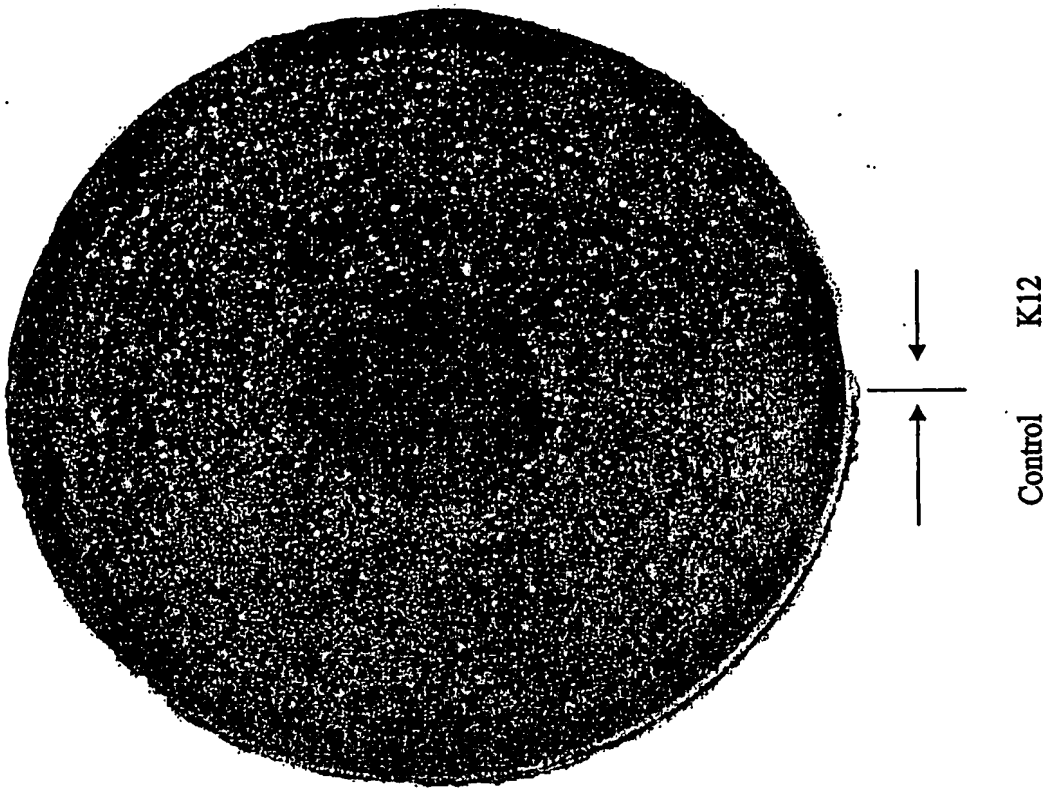
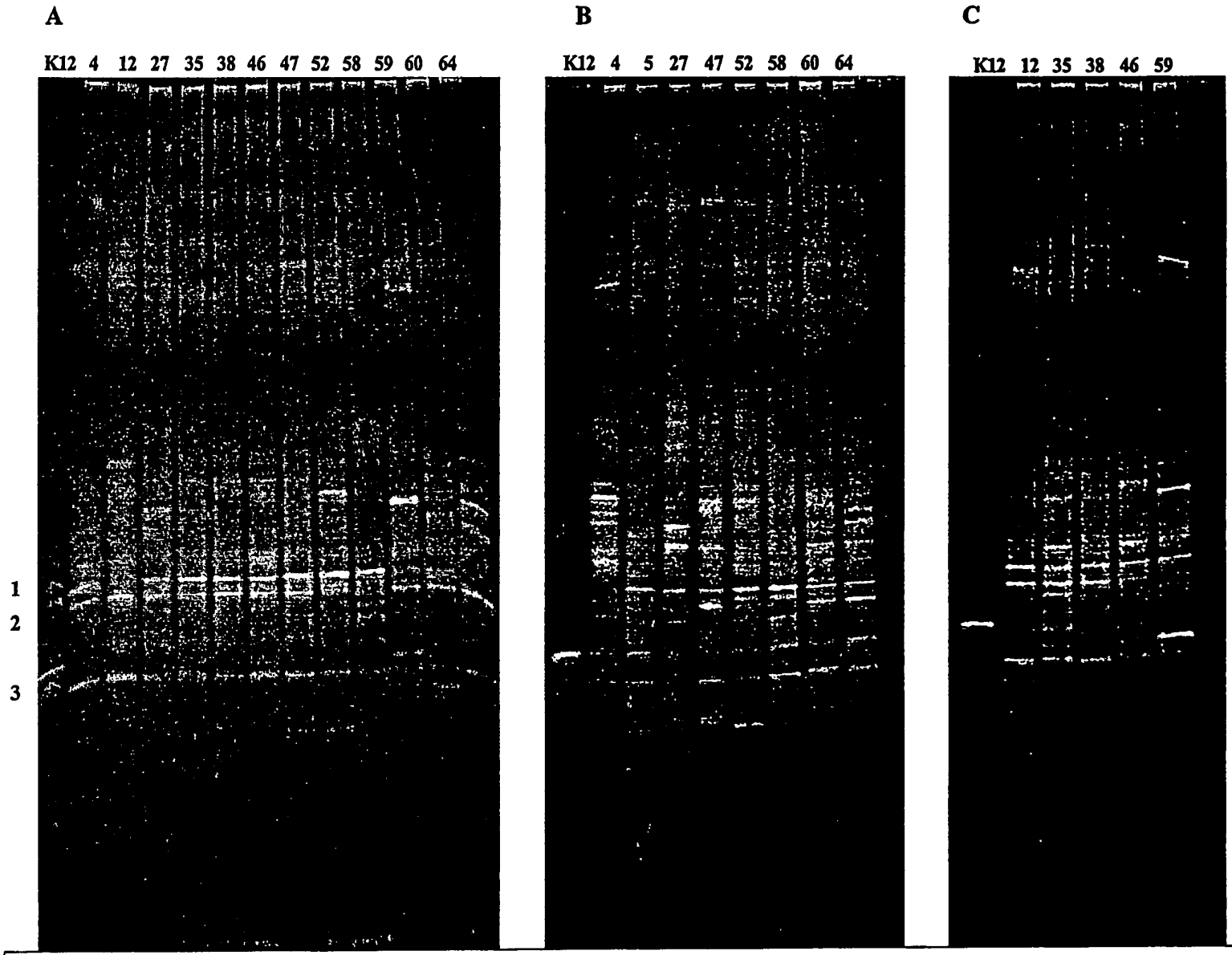


Figure 1

Figure 2



Each fragment represents bacterial strain, species or group

Figure 3

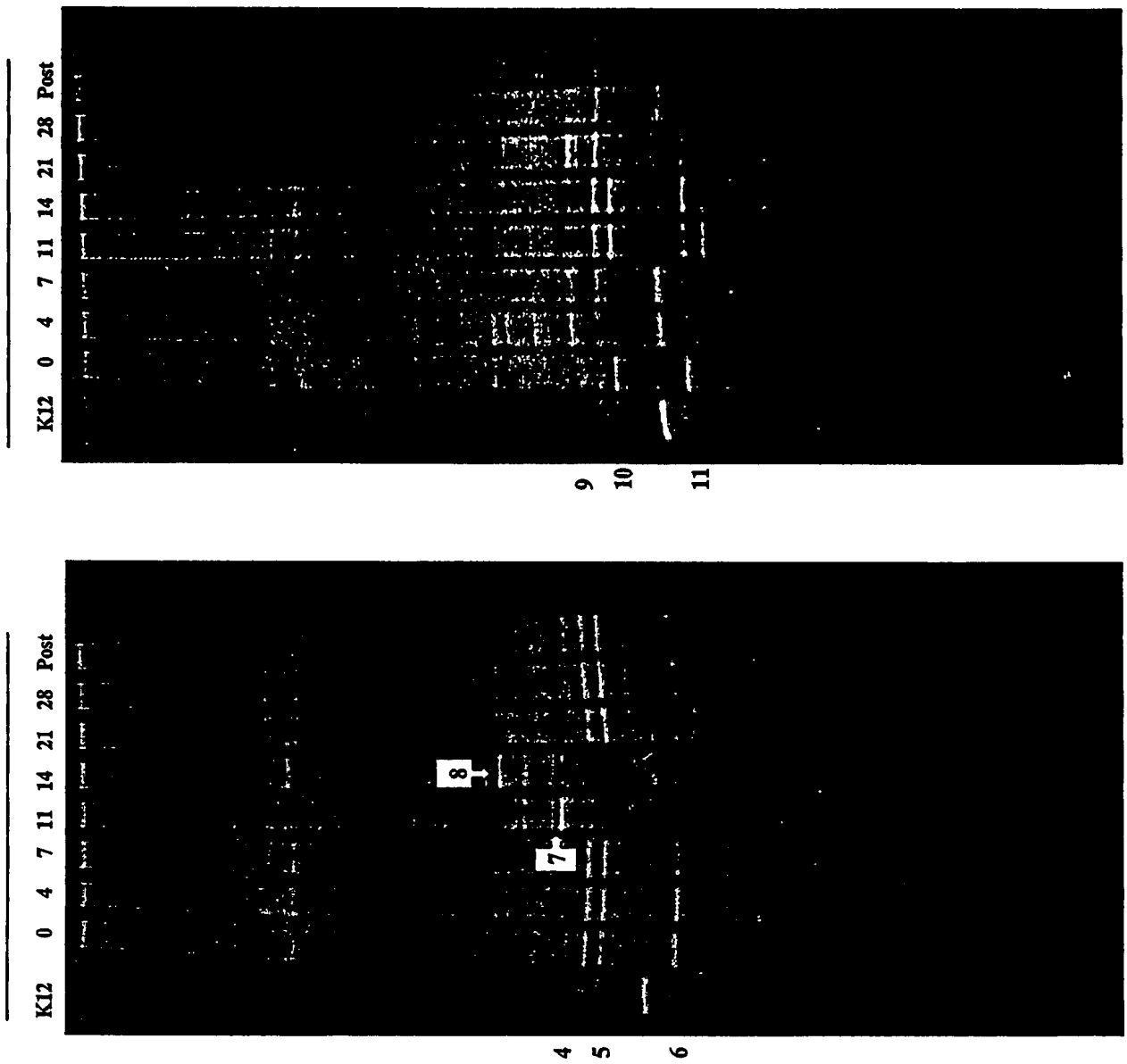
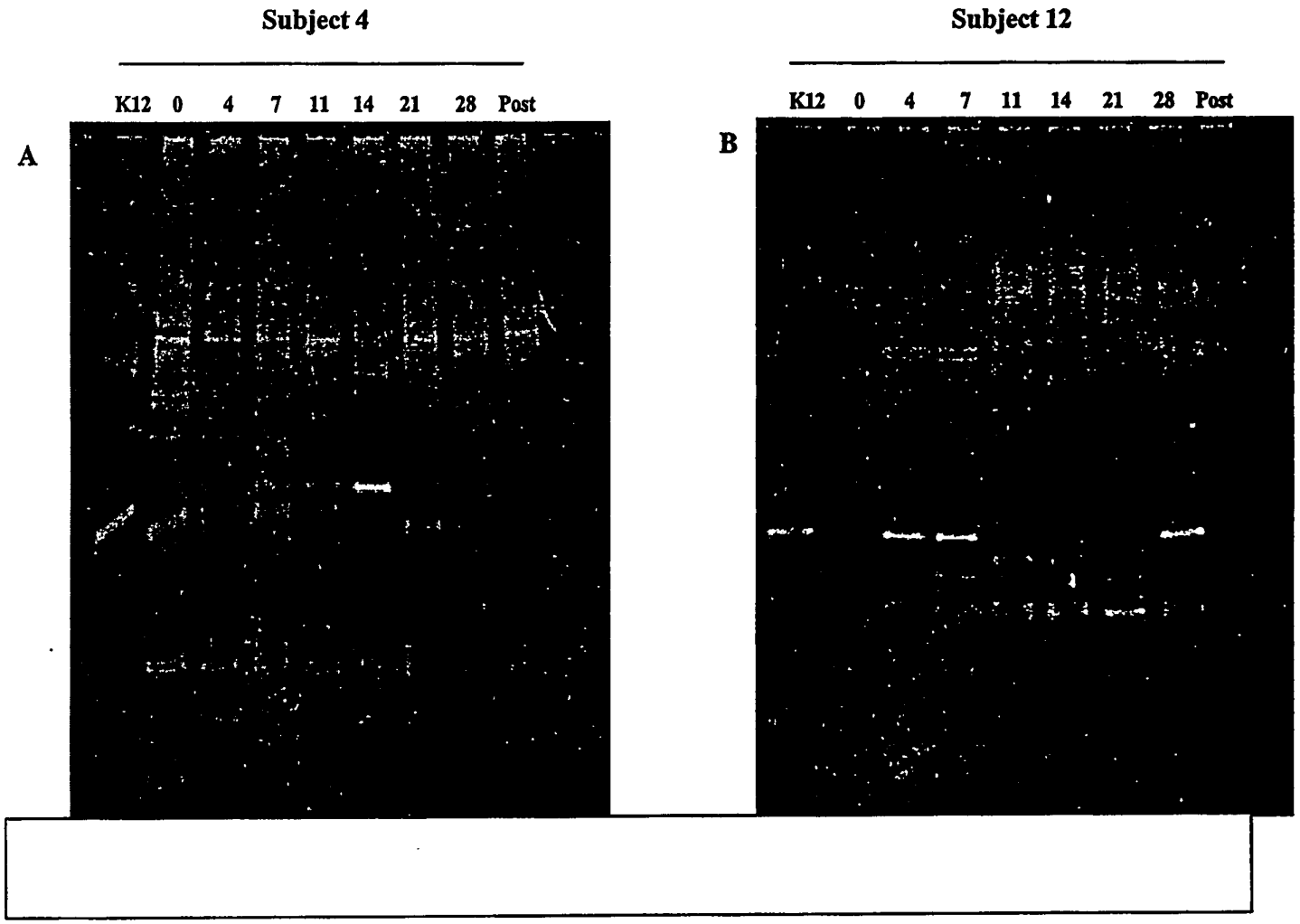
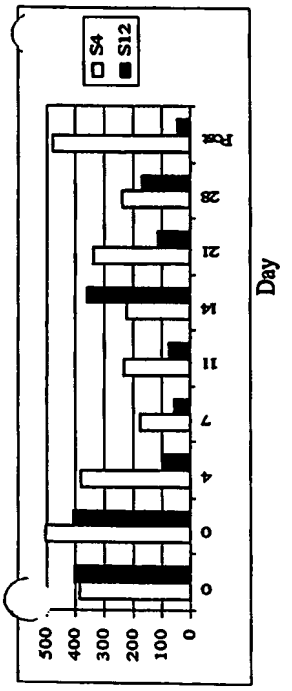


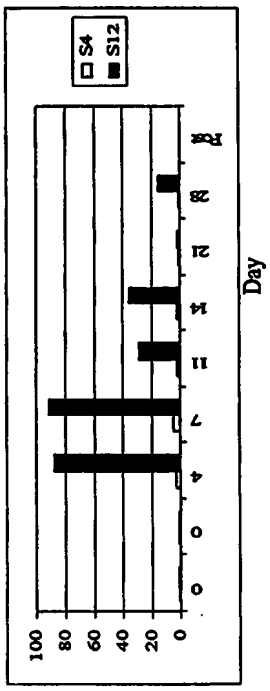
Figure 4





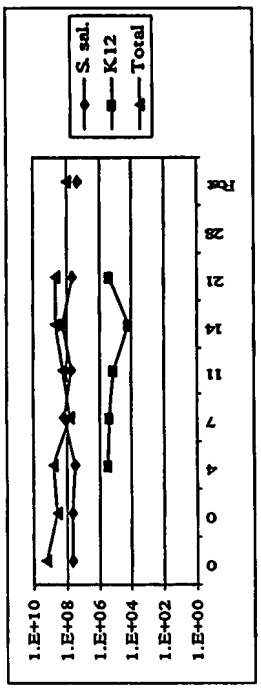
VSC (ppb)

Figure 5A



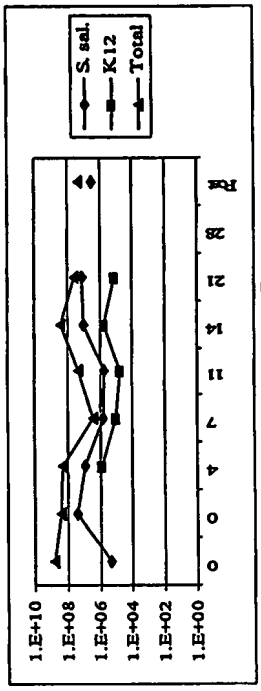
Il Activity (%)

Figure 5B



CFU/ml

Figure 5C



CFU/ml

Figure 5D